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Wnt-Planar Cell Polarity signaling controls the anterior-posterior organization of monoaminergic axons in the brainstem

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Abstract

Monoaminergic neurons (serotonergic (5HT) and dopaminergic (mdDA)) in the brainstem project axons along the anterior-posterior axis. Despite their important physiological functions and implication in disease, the molecular mechanisms that dictate the formation of these projections along the anterior-posterior axis remain unknown. Here we reveal a novel requirement for Wnt/planar cell polarity signaling in the anterior-posterior organization of the monoaminergic system. We find that 5HT and mdDA axons express the core planar cell polarity components *Frizzled3*, *Celsr3* and *Vangl2*. In addition, monoaminergic projections show anterior-posterior guidance defects in *Frizzled3*, *Celsr3* and *Vangl2* mutant mice. The only known ligands for planar cell polarity signaling are Wnt proteins. In culture, Wnt5a attracts 5HT but repels mdDA axons and Wnt7b attracts mdDA axons. However, mdDA axons from *Frizzled3* mutant mice are unresponsive to Wnt5a and Wnt7b. Both Wnts are expressed in gradients along the anterior-posterior axis, consistent with their role as directional cues. Finally, *Wnt5a* mutants show transient anterior-posterior guidance defects in mdDA projections. Furthermore, we observe that the cell bodies of migrating descending 5HT neurons eventually re-orient along the direction of their axons. In *Frizzled3* mutants, many 5HT and mdDA neuron cell bodies are oriented abnormally along the direction of their aberrant axon projections. Overall, our data suggest that Wnt/planar cell polarity signaling may be a global anterior-posterior guidance mechanism that controls axonal and cellular organization beyond the spinal cord.

Keywords

Axon guidance; development; anterior-posterior; ascending; descending; serotonin

Introduction

Axon guidance cues control the direction of growth cone navigation along the major anatomical axes (anterior-posterior (A-P), dorsal-ventral and inferior-superior) during neural circuit development. Wnt family proteins are evolutionary conserved A-P guidance cues

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(Zou, 2006; Zou and Lyuksyutova, 2007). In the vertebrate spinal cord, Wnts provide directional cues for both ascending sensory axons and descending motor axons (Lyuksyutova et al., 2003; Liu et al., 2005; Wolf et al., 2008). In contrast, the A-P axon guidance cues that function in the vertebrate CNS more rostral to the spinal cord remain largely unknown.

Monoaminergic neurons (serotonergic (5HT) and dopaminergic (mdDA)) in the brainstem control many aspects of nervous system functioning and their malfunctions are involved in the pathogenesis of a large number of nervous system diseases, such as depression, autism, and Parkinson's disease (Pardo and Eberhart, 2007; Van den Heuvel and Pasterkamp, 2008). These neurons are organized in discrete nuclei in the midbrain (mdDA; A9, A10) and hindbrain (5HT; B1-B9) and project axons along the A-P axis to reach their final targets in the brain and spinal cord (Rubenstein, 1998; Goridis and Rohrer, 2002; Cordes, 2005; Smidt and Burbach, 2007; Van den Heuvel and Pasterkamp, 2008). The A-P projections of 5HT and mdDA neurons are established during midgestation and are essential for appropriate monoaminergic circuit formation during subsequent developmental stages. Surprisingly little is known about the genetic control of monoaminergic pathway development, particularly how monoaminergic projections are initially established along the A-P axis.

One of the Wnt signaling pathways, the planar cell polarity (PCP) pathway, is involved in tissue morphogenesis and directed cell migration (Wang and Nathans, 2007; Zallen, 2007; Goodrich, 2008; Simons and Mlodzik, 2008). Core PCP components include *Frizzled*, *Flamingo* (Celsrs in vertebrates), *Van Gogh*, *Disheveled*, *Prickle* and *Diego*. In this study, we found that Wnt/PCP signaling is essential for the A-P organization of 5HT and mdDA axons in the brainstem. Both 5HT and mdDA neurons expressed core PCP components and showed A-P guidance defects in *Frizzled3*, *Celsr3* and *Vangl2* (*Looptail*) mutant mice. In addition, *Wnt5a* mutants showed transient A-P guidance defects for mdDA axons. *Wnt5a* and *Wnt7b* were expressed in gradients along the A-P axis in the brainstem, consistent with their role as directional cues. In culture, *Wnt5a* attracted 5HT axons but repelled mdDA axons, whereas *Wnt7b* attracted mdDA axons. In contrast, dopaminergic neurons derived from *Fzd3* mutant mice did not respond to *Wnt5a* or *Wnt7b*. Analyses of wildtype or *Fzd3* mutant mice further revealed that cell bodies of descending 5HT neurons are initially oriented medial-laterally and gradually shift to orient along the A-P axis following the direction of their axons and that proper Wnt/PCP signaling is essential for this A-P cellular organization of monoaminergic nuclei. Together these results define novel and crucial roles for Wnt/PCP signaling in establishing the A-P axonal and cellular organization of monoaminergic systems. Also, these findings indicate that A-P axon guidance functions of different PCP proteins are not limited to the spinal cord but function more globally in the vertebrate CNS.

Materials and Methods

Animals

All animal use and care were in accordance with institutional guidelines. CD-1 and C57BL/6 mice were obtained from Charles River. *Fzd3* mutant mice were obtained from Jeremy Nathans (Johns Hopkins University School of Medicine, Baltimore, USA (Wang et al., 2002)), *Looptail* mice from Tony Wynshaw Boris (UCSF, San Francisco, USA (Montcouquiol et al., 2006)), and *Ryk* mutant mice from Steven Stacker (Ludwig Institute for Cancer Research, Australia (Halford et al., 2000)). *Celsr3* mutant mice have been described before (Tissir et al., 2005). *Wnt5a* mutant mice were purchased from Jackson Laboratories. Timed-pregnant mice were killed by means of cervical dislocation. The morning on which a vaginal plug was detected was considered embryonic day 0.5 (E0.5). For *in situ* hybridization and immunohistochemistry E11.5-E17.5 brains were fixed in 4%

paraformaldehyde (PFA) for 30 minutes to 1 hour, followed by immersion in 30 % sucrose. Brains were frozen on powdered dry ice, cryosectioned, mounted on Superfrost Plus slides (Thermo Fisher Scientific), air-dried, and stored desiccated at -80°C.

In situ hybridization and immunohistochemistry

Non-radioactive *in situ* hybridization on cryosections (14 μ m) was performed as described previously (Pasterkamp et al., 2007). Specific digoxigenin-labeled *in situ* probes for the mouse Wnt family were as described (Liu et al., 2005). Whole mount *in situ* hybridization was performed as described previously (Henrique et al., 1995).

Cryosections (14 μ m) were stained immunohistochemically as described previously (Kolk et al., 2009). Sections were counterstained with fluorescent Nissl (1:500; Neurotrace, Invitrogen) or DAPI (Sigma), washed extensively and embedded in Mowiol (Sigma). Staining was visualized using a Zeiss Axioskop 2 microscope or by confocal laser-scanning microscopy (Zeiss LSM510). The following primary antibodies were used: rabbit anti-Frizzled3 (1:500, a gift from Jeremy Nathans), rabbit anti-Celsr3 (1:500, generated in the Zou lab), goat anti-Vangl2 (1:100, Santa Cruz), rat anti-5HT (1:500; Accurate Chemicals), rabbit anti-TH (1:1000, Pel-Freeze), and mouse anti-TH (1:500, Immunostar).

TH Explant assays

Three-dimensional collagen matrix assays using SN and VTA explants were performed as described previously (Kolk et al., 2009). SN or VTA explants were rapidly dissected from E12.5-E14.5 C57BL/6 embryos or from wildtype or *Frizzled3* mutant embryos and embedded in close proximity to (~300 μ m apart) aggregates of HEK293 cells transiently transfected with expression vectors for Wnt5a or Wnt7b, or mock-transfected as a control. Explants were cultured for 56-58 h, fixed and co-immunostained using rabbit anti-TH (1:1000; Pel-Freeze) and mouse anti- β III-tubulin (1:3000; Covance) antibodies to stain dopaminergic and all neurites emerging from the explants, respectively.

For quantification of explant assays, the length of the 20 longest TH positive neurites was measured in both the proximal and distal quadrants of the explant cultures using OpenLab software (Improvision). The average value of the 20 neurites proximal and distal was used to determine the proximal/distal ratio (P/D ratio) per explant. Data were statistically analyzed by one-way ANOVA ($\alpha=5\%$) and expressed as means $\pm$ SEM.

5HT open book explants

Hindbrains were dissected from E12.5 time pregnant CD-1 mice in L15 media and placed ventricular zone down onto a bed of collagen. Blue sepharose beads previously coated overnight at 4°C in 100 ng/ μ l of purified Wnt proteins were placed along the midline of the explants and then the explant was covered in a top layer of collagen. Explants were incubated at 37°C for 24 h and fixed with 4% PFA overnight at 4°C. 5HT positive neurons were visualized using whole mount immunostaining with rat anti-5HT (1:300; Accurate Chemicals). Images were acquired by confocal microscopy and Z stacks were compressed and analyzed in ImageJ.

For the quantification of hindbrain open book cultures, we counted the number of 5HT positive axons emanating from the midline of the ascending population. The axons were grouped as either growing anteriorly, laterally, or posteriorly. The number of axons growing anteriorly was graphed as a percentage of the total number of axons counted. Over 100 axons were counted for each explant (control n=10, Wnt4 n=6, Wnt5a n=10, Wnt7a n=9). Data was statistically analyzed by one-tailed student T-Test and represented as $\pm$ SEM.

Quantification of premature axon invasion into rhombomere four

For quantification of the axon guidance defect of the ascending 5HT population, we measured the length of axons past the start of the descending neuron population (see arrowhead in Fig 2 G-J). Both *Fzd3* and *Lp* mice were compared with littermate controls as there were developmental differences among different genetic backgrounds (B6 mixed versus Sv129J mixed). This analysis was used to show that the mutants invaded the region below rhombomere 4 before their wild type counterparts did. (*Fzd3* $+/+$ $n=6$, *Fzd3* $-/-$ $n=4$, *Lp* $+/+$ $n=4$, *Lp/Lp* $n=4$). Data was statistically analyzed by one-tailed student T-Test and represented as $\pm$ SEM.

Dissociated neuron cultures

To generate dissociated neuron cultures from the midbrain, the mdDA system was microdissected from E14.5 C57BL/6 wildtype embryos. Tissue from several different embryos was pooled and incubated in trypsin for 20 min at 37°C. Trypsin activity was inhibited with 20% fetal calf serum. Then, the cells were pelleted by mild centrifugation and resuspended in Neurobasal Medium supplemented with B27, pencillin/streptomycin, and glutamine. A single cell suspension was generated by putting the cells through a 70 μ m filter after which cells were plated on poly-D-lysine- and laminin-coated glass coverslips in a 12-well plate for 2-3 days. Hindbrain neuron cultures were generated similar to dopaminergic neurons but with the following modifications. Hindbrains were dissected anterior to rhombomere 4 from E12.5 CD1 mouse embryos. Tissue from multiple embryos was pooled in incubated in 0.025% trypsin for 20 min at 37°C. The tissue was then triturated with fire polished glass pasture pipets and trypsin activity was inhibited by the addition of RPMI plus 5% horse serum. Cells were plated in Neurobasal media as above on poly-D-lysine- and laminin-coated glass coverslips and incubated for 2 days. All cells were fixed with 4% paraformaldehyde for 10 min at room temperature and immunostained with the following primary antibodies: rabbit anti-Frizzled3 (1:500, a gift from Jeremy Nathans), rabbit anti-Celsr3 (1:500), goat anti-Vangl2 (1:100, Santa Cruz), rat anti-5HT (1:500; Accurate Chemicals), rabbit anti-TH (1:1000, Pel-Freez), and mouse anti-TH (1:500, Immunostar).

Quantification cell body orientation

To assess and quantify mdDA cell body orientation defects in *Fzd3* mutant mice, sagittal sections from E12.5 *Fzd3* $-/-$ embryos ($n=3$) and littermate controls ($n=3$) were immunostained with anti-TH antibodies and counterstained with DAPI. Images through the lateral and medial midbrain region were captured from these sections at identical medial to lateral locations using a Zeiss Axioskop 2 microscope. Then, using Zeiss Axiovision software, angles were determined between the lining of the mesencephalic flexure (baseline, represents 0°) and the trajectory of the initial axon segment just proximal to the TH positive cell body. 50-100 TH-positive neurons were measured per embryo at the lateral and medial levels and angles were grouped (0/45, 45/90° etc) and calculated as percentage of total.

To analyze the orientation of 5HT cell bodies of the descending population, we imaged whole mount immunostaining of 5HT neurons using anti-5HT antibodies from E12.5 *Fzd3* $-/-$ ($n=3$) and wildtype littermate controls ($n=3$). Z-stack images were acquired by confocal microscopy and analyzed with Image J. Cell body orientation was assessed by drawing a line vertically down to the center of the cell followed by drawing an intersecting line through the center of the initial segment of the leading process. The angle of this intersection was measured in Image J. Using this method, a completely horizontal cell would give an angle of 90° while a completely vertical cell would yield an angle of 180°. Over 100 5HT positive cells were counted per embryo and the angles were binned (0/45, 45/90° etc) and graphed as percentage of total as for TH quantification. Data were statistically analyzed by one-tailed student T-Test and represented as $\pm$ SEM.

Results

Serotonergic and dopaminergic neurons express core PCP components

In mouse, ascending 5HT neurons are born between E11.5 and E12.5, just posterior to the isthmus and are located close to the midline (Fig. 1A, B, upper red oval). They reside in 6 different nuclei (B4-B9) and send projections anteriorly to innervate various CNS structures. Shortly after the appearance of the ascending 5HT neurons, descending 5HT neurons migrate laterally in rhombomeres 5-7, become immunoreactive for 5-hydroxytryptamine (5HT), and begin projecting axons posteriorly by E12.5 (Fig. 1A, B, lower red oval). These neurons reside in 3 distinct nuclei (B1-B3) and project axons into the cervical spinal cord. Despite the well-characterized anatomy of the 5HT system, its axon guidance mechanisms, particularly along the A-P axis, are unknown. The first mesodiencephalic dopamine (mdDA) neurons, located just anterior to the isthmus, are born at approximately E10.5 and start to extend axons at E11.5. The mdDA system is composed of a medial population close to the midline, the ventral tegmental area (VTA, A10), and a lateral subdivision, the substantia nigra (SN, A9) (Fig. 1A, B, green oval) and both give rise to prominent ascending (anterior) axonal projections to the striatum and the medial prefrontal cortex.

It is evident that robust mechanisms must be in place to guide both hindbrain 5HT and midbrain mdDA axons into their normal A-P directions. Wnts and their receptors have been implicated in determining the growth polarity of long descending and ascending spinal axon projections (Lyuksytova et al., 2003; Liu et al., 2005; Wolf et al., 2008). In addition, Wnts and their receptors are expressed in the midbrain during early embryonic development to regulate neuronal proliferation and differentiation (Andersson et al., 2008). Furthermore, in mice lacking *Frizzled3* (*Fzd3*) dopaminergic innervation of the striatum is lost (Wang et al., 2002). These results suggest that *Fzd3* and associated signaling proteins may act to guide the growth of monoaminergic axons.

To test this hypothesis, we first examined the expression of core PCP components in the developing hindbrain by co-immunostaining against PCP proteins and 5HT. We examined sagittal sections of the mouse hindbrain at E11.5 (Fig. S1) and E12.5, stages at which 5HT neurons are born and begin pathfinding. We found that several PCP core components, *Fzd3*, *Vangl2*, and *Celsr3*, are expressed at E11.5 and E12.5 in both the ascending and descending 5HT populations in cell bodies and fibers (Fig. 1C). Most, if not all, 5HT-positive cells expressed *Fzd3*, *Vangl2* and *Celsr3*. We also performed dissociated neuron cultures followed by immunocytochemistry and found that all 5HT-positive cells express *Fzd3* (Fig. 1D) and *Celsr3* (Fig. 1E). To examine the expression of core PCP components in mdDA neurons, we used co-immunostaining against PCP proteins and TH on coronal sections. We focused on E11.5 to E14.5, during which mdDA axon growth polarity is established. *Fzd3*, *Celsr3* and *Vangl2* are widely expressed in the midbrain region and in all TH positive mdDA neurons as exemplified by E14.5 (Fig. 1F). Using dissociated neuron culture and immunocytochemistry, we confirmed that all TH-positive cells express *Fzd3* and *Celsr3* (Fig. 1G, H). Unfortunately, the *Vangl2* antibody did not stain well in the dissociated cultures. However, the immunostaining in Fig. 1C and 1F clearly shows the presence of *Vangl2* in 5HT and TH neurons.

PCP signaling is required for A-P guidance of serotonergic axons

To examine the potential role of PCP signaling in the development of the 5HT system, we analyzed mouse lines deficient in PCP signaling components (Fig. 2, 3). Whole mount immunostaining against 5HT in the hindbrain reveals both the cell bodies and axons of serotonergic neurons (Fig. 2, 3). The *Fzd3* ^{-/-} mouse (Wang and Nathans, 2007) displays dramatic axon guidance defects in the 5HT system at E12.5 (Fig. 2A-C). Although 5HT

positive cell bodies appear to be in the appropriate A-P positions in *Fzd3* ^{-/-} mice, the axons in both the ascending and descending populations display marked misprojections. Axons in the ascending population project posteriorly and laterally instead of strictly anteriorly (Fig. 2C, 2H, 3B). At E12.5, when there are normally no axons present in rhombomere 4 (R4), axons erroneously descend well into this rhombomere (Fig. 2C, F, H). The descending 5HT population also displays striking axon guidance defects. Descending axons are distinctly shorter than their wild type counterparts and project randomly along the A-P axis (Fig. 2C).

The *Looptail* mouse carries a mutation in the *Vangl2* protein that greatly affects *Vangl2* stability and displays gross PCP defects (Montcouquiol et al., 2006). At E12.5 the *Lp* mouse displays axon guidance defects in both the ascending and descending populations (Fig. 2D, 2I, 3C). The ascending population displays a premature invasion of R4 and posterior projections (Fig. 2D, 2F, 2I). The descending population displays a striking over-fasciculation defect that is not present in the other PCP mutants we analyzed. Many of these axons grow together in tight bundles as if they were attracted to each other and appear much shorter (Fig. 2D). Higher magnification images further reveal that these axons appear highly fasciculated (Fig. 3F, G). Like *Fzd3* mutants, very few axons project posteriorly into the cervical spinal cord in *Lp* mice.

We next examined 5HT axon projections in *Celsr3* ^{-/-} mice (Tissir et al., 2005). Like the *Fzd3* ^{-/-} mouse, ascending axons in *Celsr3* ^{-/-} mice misproject both posteriorly and laterally (Fig. 2E, arrowhead). Axons also invade the R4 region at E12.5 although not as prominently as in *Fzd3* mutants (Fig. 2E, 2J). Although the descending population appears to show some orientation defects as observed in the *Fzd3* ^{-/-} mouse, many neurons form normal descending projections (Fig. 2E). This is in sharp contrast to the *Fzd3* ^{-/-} mouse, in which very few descending axons are formed at this stage.

At higher magnifications, we found that the cell bodies of wildtype neurons in the descending population are first oriented laterally while migrating following the direction of their leading axons but eventually become oriented along the A-P axis as their axons turn posteriorly (Fig. 3H-J, N). However, in *Fzd3* ^{-/-} mice many of the descending, but not ascending, cell bodies appear to remain oriented laterally or even slightly anteriorly. These cell bodies give rise to axons that grow out laterally before turning anterior or posterior (Fig. 3K, 3L, 3O). At E12.5, almost 50% of descending 5HT neurons in *Fzd3* ^{+/+} mice are oriented at 180° compared to 10% in *Fzd3* ^{-/-} mice (Fig. 3P). Because during normal development axons are first oriented along the A-P axis before the soma rotates into its A-P position, it is plausible that the cell body follows the axon and uses it to position itself along the A-P axis (see also Hawthorne et al., 2010). Although our observations strongly support this model, we currently cannot exclude the possibility that PCP signaling may also regulate soma and dendrite orientation independent from A-P axon guidance.

Analysis of mice deficient for Ryk (Halford et al., 2000), another Wnt receptor, did not reveal obvious defects (Fig. S2). Similarly, genetic inactivation of Wnt5a (Yamaguchi et al., 1999), a known PCP Wnt, also did not appear to have a striking effect on 5HT A-P axon guidance (Fig. S2).

PCP signaling is required for A-P guidance of dopaminergic axons

To assess whether Wnt/PCP signaling also controls mdDA axon patterning, we visualized mdDA axon trajectories in sections from *Fzd3* ^{-/-} mice using TH immunohistochemistry. At E11.5, many mdDA axons in *Fzd3* ^{-/-} mice follow an abnormal lateral trajectory at the level of the mdDA neuron pool (Fig. S3). At E12.5 and E14.5, a large number of mdDA axons are directed posteriorly in *Fzd3* ^{-/-} mice and aberrantly traverse the hindbrain (Fig. 4A-D). Although a subset of mdDA axons still projects anteriorly, presumably due to the presence

of other A-P signals (Kolk et al., 2009; Yamauchi et al., 2009), this anterior projection is significantly reduced and disorganized in *Fzd3* ^{-/-} mice. In addition, many mdDA axons enter abnormal dorsal territories in the midbrain and diencephalon and aberrantly approach the midline (Fig. 4E-G). In the telencephalon, mdDA axons are displaced ventrally and are located in close proximity to the optic chiasm (Fig. 4H, I). No innervation of synaptic target regions such as the striatum or medial prefrontal cortex by mdDA axons is found at E14.5 or E17.5 (Fig. 4J, K; data not shown).

Analysis of *Celsr3* ^{-/-} and *Lp* mice reveals similar abnormal posterior mdDA projections in the hindbrain at E12.5 and E17.5. At E12.5, *Fzd3* ^{-/-} and *Lp* mice show a similar, large number of posterior mdDA projections, while at this stage this defect is less pronounced in *Celsr3* ^{-/-} mice (Fig. S4). At E17.5, however, numerous mdDA axons are projecting into the hindbrain of *Fzd3* ^{-/-} and *Celsr3* ^{-/-} mice, but fewer posterior projections are present in *Lp* mice (Fig. 4L-O). In the diencephalon and telencephalon of *Celsr3* ^{-/-} mice, many mdDA axons follow aberrant lateral, dorsal and ventral trajectories, as observed in *Fzd3* ^{-/-} mice. In contrast to *Fzd3* ^{-/-} mice, however, a small subset of mdDA axons reaches but does not innervate the striatum in E17.5 *Celsr3* ^{-/-} mice (Fig. S5A, B). MdDA axon projections in the forebrain of E17.5 *Lp* mice appear to innervate the striatum. However, it should be noted that *Lp* mice have an open neural tube and that the overall organization of the telencephalon is markedly changed in these mice (data not shown).

We also examined mdDA axon projections in *Wnt5a* ^{-/-} mice. In contrast to *Fzd3* ^{-/-}, *Celsr3* ^{-/-} and *Lp* mice, *Wnt5a* ^{-/-} mice only display a minor and transient posterior projection of mdDA axons (Fig. 4P; inset). This suggests that the lack of Wnt5a signaling is compensated by other Wnt proteins at later developmental stages. We also analyzed mice deficient for the Wnt5a receptor Ryk. *Ryk* ^{-/-} mice did not show overt A-P guidance defects (Fig. 4Q). In addition, mdDA innervation of the striatum appears grossly normal at E17.5 in both *Wnt5a* ^{-/-} and *Ryk* ^{-/-} mice (Fig. S5C-F).

The descending 5HT system shows defects in cell body orientation in *Fzd3* ^{-/-} embryos, which may be secondary to defective axon guidance (Fig. 3). Intriguingly, analysis of mdDA neurons at E11.5 to E14.5 reveals similar defects. At E11.5, the overall cellular organization of the mdDA system is normal in *Fzd3* mutants (Fig. S3). At E12.5, mdDA cell bodies and axons in the most lateral aspect of the mdDA neuron pool of wildtype mice are oriented anteriorly (Fig. 5A-D, G). In contrast, mdDA cell bodies at the same anatomical location in *Fzd3* ^{-/-} mice are oriented in random directions following where the axons are oriented (Fig. 5E, F, G). mdDA neurons in the medial part of the mdDA system of wildtype mice send their axons dorsally before these axons are redirected anteriorly (Nakamura et al., 2000) (Fig. 5H, I, L). In contrast to lateral mdDA neurons, mdDA neurons in the medial part of the mdDA neuron pool in *Fzd3* ^{-/-} mice do not display obvious defects in cell body orientation (Fig. 5J-L). This difference between the lateral and medial mdDA neuron pool is reminiscent of what is observed for 5HT neurons, with descending (lateral) but not ascending (medial) neurons showing cell body orientation defects (Fig. 3).

Wnts are expressed in anterior-posterior gradients in the basal midbrain and hindbrain

The only known ligands for PCP signaling are Wnt proteins. Using *in situ* hybridization, we sought to determine whether Wnts are expressed in the hindbrain and midbrain region. Several Wnts were expressed in the developing hindbrain but *Wnt5a* displayed the most striking expression pattern. In the hindbrain, we found that *Wnt5a* is expressed in an anterior high posterior low expression pattern at the midline where ascending serotonergic neurons are projecting anteriorly (Fig. 6A, B). Interestingly, the *Wnt5a* gradient direction switched from decreasing in the posterior direction to increasing at rhombomere 4 (Fig. 6A, I). It is therefore possible that Wnt5a attraction contributes to the proper A-P guidance of both

ascending and descending 5HT axons. In the midbrain of E12.5 and E14.5 embryos we identified clear expression gradients for *Wnt5a* and *Wnt7b*. *Wnt5a* labels the medial part of the mdDA system (VTA) and displays a high posterior low anterior expression gradient with highest expression around the isthmus (Fig. 6C, D, F, G; (Andersson et al., 2008)). This *Wnt5a* gradient flips at the isthmus and starts to decrease towards more posterior regions (Fig. 6I). *Wnt7b* is expressed in both the medial and lateral mdDA system and shows a high anterior low posterior expression pattern in the midbrain (Fig. 6E, H). In all, these experiments show that Wnt proteins form expression gradients in the hindbrain and midbrain at the time 5HT and mdDA axons are guided into the appropriate A-P direction.

Wnt proteins influence the orientation of 5HT and mdDA axons and cell bodies

Because Wnts are expressed in gradients in the mid/hindbrain region, we tested whether Wnt proteins can direct the growth of 5HT and mdDA axons. To determine if Wnt ligands can direct 5HT axons, we established a new hindbrain “open-book” explant culture. We added Wnt coated beads along the midline to disrupt endogenous Wnt gradients (Fig. 7A). The exogenous presence of Wnt4, Wnt5a, and Wnt7a all affected the ability of axons in the ascending population to grow anteriorly towards the brain (Fig. 7B-7D). Many of these axons now grew into the midline, posteriorly, or laterally, and fewer axons projected anteriorly (see blue misprojected axons in Fig. 7D). Some cultures also displayed 5HT axons and cell bodies that were attracted to the midline after the addition of Wnt coated beads (Fig. 7D). These defects correlate well with the misguidance phenotypes of ascending 5HT neurons in PCP mutant mice. The placement of the Wnt coated beads did not appear to have a robust effect on the descending population probably due to that fact that the descending axons and cell bodies are too far away from the midline, the site of bead implantation. These experiments suggest that Wnt5a serves as an attractive guidance cue for ascending 5HT axons (Fig. 6I). To determine whether Wnts instruct mdDA axons to grow anteriorly we used a previously established collagen matrix culture approach (Kolk et al., 2009). The chemotropic response of mdDA axons emanating from E12.5 SN or VTA explants to different Wnt proteins was tested. When confronted with cell aggregates releasing Wnt5a, TH positive SN and VTA axons displayed strong chemorepulsive responses (Fig. 7F, 7I). In contrast, Wnt7b induced axon attraction of both mdDA axon populations (Fig. 7G, 7I). When examining all axons emanating from SN and VTA explants, as visualized by β III tubulin staining, no chemotropic effects were found confirming the specificity of the response of TH positive axons to Wnt5a and Wnt7b (Fig. 7H). These results together with the Wnt expression analysis suggest that Wnt5a functions to repel and Wnt7b to attract mdDA axons into their normal anterior direction in the midbrain (Fig. 6I).

Dopaminergic axons from *Fzd3* mutant mice are unresponsive to Wnts *in vitro*

Our results show expression of core PCP components in 5HT and mdDA neurons and axon projections (Fig. 1) and reveal A-P guidance defects in 5HT and mdDA axon pathways in PCP mutant mice *in vivo* (Fig. 2-4). The only known PCP ligands, Wnt proteins, are expressed in instructive gradients in the brainstem and provide directional cues for 5HT and mdDA axons *in vitro* (Fig. 6, 7). Together, these data support the idea that PCP proteins serve as Wnt receptors on monoaminergic axons during A-P guidance. To further test this model, we performed collagen matrix assays with VTA explants dissected from *Fzd3* wildtype or mutant embryos. *Fzd3* had been implicated as a neuronal receptor for Wnt3a and Wnt4 (Lyuksyutova et al., 2003) (Endo et al., 2008). As described above, dopaminergic axons from wildtype explants were repelled by Wnt5a and attracted by Wnt7b (Fig. 8A, C, E, G). In contrast, axons emanating from dopaminergic explants obtained from *Fzd3*^{-/-} mice did not display significant repulsive or attractive responses to Wnt5a and Wnt7b, respectively, as compared to wildtype and knockout control cultures (Fig. 8B, D, F, G). These results show that *Fzd3* is an obligatory component of the receptor for Wnt5a and

Wnt7b on dopaminergic axons and support the model that PCP receptors on monoaminergic axon projections function to sense instructive Wnt gradients in the brainstem thereby controlling A-P axon guidance.

Discussion

Serotonergic and dopaminergic axon pathways mediate complex physiological functions and are therapeutic targets for many nervous system disorders. Their organization is exquisitely complex and requires a myriad of molecular signals for regulating different developmental steps. A crucial step in establishing monoaminergic connectivity is the initial orientation of 5HT and mdDA axon projections along the A-P axis of the brainstem. Here we reveal a novel requirement for Wnt and PCP receptor proteins in the A-P guidance of 5HT and mdDA axon projections. In PCP signaling mutants, both 5HT and mdDA axons are severely misguided along the A-P axis (Fig. 9). Furthermore, we show that A-P cellular organization (i.e. cell body orientation) of monoaminergic nuclei is affected in Wnt/PCP mutants, without marked defects in the patterning of the brainstem (Fig. S6).

Wnt signaling controls A-P axon guidance beyond the spinal cord

Wnt family proteins have been identified as guidance cues that pattern axonal connections along the A-P axis in vertebrates and invertebrates (Zou, 2006; Zou and Lyuksyutova, 2007; Salinas and Zou, 2008). In vertebrate species, Wnts provide directional cues for ascending sensory and descending corticospinal axons along the A-P axis of the spinal cord (Lyuksyutova et al., 2003; Liu et al., 2005; Wolf et al., 2008). Until now it was unknown whether A-P guidance in parts of the CNS outside the spinal cord also required Wnt signaling. In the present study, we show that indeed A-P guidance of 5HT and mdDA axons depends on Wnt signaling. Both 5HT and mdDA neurons and axons expressed PCP components (Fig. 1) and mice lacking these proteins displayed prominent monoaminergic A-P guidance defects in the midbrain and hindbrain (Fig. 2-4, 9). The only known PCP receptor ligands, Wnt proteins, were expressed in gradients in the brainstem and mediated guidance responses from 5HT and mdDA axons in culture (Fig. 6, 7). mdDA neurons lacking the PCP receptor *Fzd3* did not show significant axonal responses to Wnt5a and Wnt7b in culture (Fig. 8). These results establish that Wnt signaling through PCP pathway is a global A-P guidance mechanism that patterns axon tracts both in and outside the spinal cord. The marked defects in mdDA axon projections found in the telencephalon of *Fzd3* and *Celsr3* mutant mice (Fig. 4) support the idea that Wnt signaling may also control A-P guidance in the forebrain. In future studies, it will therefore be interesting to explore whether mdDA pathways or other longitudinal axon bundles in the forebrain are organized along the A-P axis by Wnt signaling.

Complex modes of Wnt signaling during A-P guidance in the brainstem

Our study also reveals the remarkable complexity of Wnt signaling in the brainstem. Wnt gradients in the brainstem are not unidirectional and the same Wnt gene (e.g. *Wnt5a*) can display opposite gradients anterior or posterior to morphologically recognizable boundary structures, like the isthmus, or morphologically non-recognizable but molecularly defined structures, like rhombomere 4 (Fig. 6). Furthermore, the same mdDA neurons can respond differently to different Wnts, which are deposited in opposite gradients in the midbrain (attraction by Wnt7b and repulsion by Wnt5a) to ensure correct A-P guidance (Fig. 7). In addition, neighboring neuronal populations (mdDA and 5HT) respond differentially to the same Wnt protein (Wnt5a repels mdDA axons but attracts 5HT axons) (Fig. 7). The molecular mechanism that underlies this difference in Wnt5a responsiveness is currently unknown but is likely to involve additional receptor proteins.

There may also be redundancy in the PCP signaling components involved in 5HT and mdDA A-P guidance. *Fzd3* mutants show broad and strong defects. In contrast, mutations in *Celsr3* and *Vangl2* result in less severe or partial phenotypes. For example, descending 5HT neurons show much weaker phenotypes in axon guidance and cell body defects in *Celsr3* $-/-$ compared to *Fzd3* $-/-$ mice (Fig. 2). Potentially, other members of the *Celsr* family, *Celsr1* or *Celsr2* (Tissir et al., 2002), compensate for the loss of function of *Celsr3*. The persistence of a small number of appropriate 5HT and mdDA A-P projections in different PCP mutants further indicates that in addition to PCP signals other molecular cues are involved in A-P guidance in the brainstem (Kolk et al., 2009; Yamauchi et al., 2009). Finally, *Looptail* mice display a severe hyperfasciculation phenotype which is not found in *Fzd3* $-/-$ and *Celsr3* $-/-$ mice suggesting that *Vangl2* may also control axon-axon interactions (Fig. 3).

Although there is a transient defect in mdDA axon projections in *Wnt5a* $-/-$ embryos, we did not observe a similar phenotype in 5HT axon projections. In addition to *Wnt5a*, *Wnt4* and *Wnt7b* are expressed in the floor plate. The *Wnt7b* and *Wnt5a* gradients follow the same direction at the level of the ascending 5HT system and both Wnts appear to attract ascending 5HT axons. Thus, *Wnt7b* may compensate for the loss of *Wnt5a* (Fig. 6). It is interesting that even though *Wnt7b* may attract mdDA axons anteriorly, we still observed a transient TH axon phenotype in the absence of *Wnt5a* (Fig. 4). It is possible that mdDA axons depend more on *Wnt5a* repulsion than *Wnt7b* attraction for anterior growth or perhaps the *Wnt5a* gradient is established first to repel mdDA axons anteriorly after which the *Wnt7b* gradient is formed slightly later to further attract mdDA axons anteriorly (Fig. 6). In this model, mdDA axon projections may eventually become corrected by the *Wnt7b* gradient in *Wnt5a* $-/-$ mice. Alternatively, aberrant axons in *Wnt5a* $-/-$ mice may not survive and die back at later developmental stages. Future studies will address these and other possible mechanisms.

Wnt/PCP signaling dictates cell body orientation along the A-P axis

Neural circuit construction involves the proper orientation of axons, dendrites and cell bodies. Because of their well-documented anatomical organization, dopaminergic and serotonergic nuclei comprise an advantageous system for studying molecular cues that establish the polarity of neurons and their projections during development (Rubenstein, 1998; Goridis and Rohrer, 2002; Cordes, 2005; Smidt and Burbach, 2007; Van den Heuvel and Pasterkamp, 2008). In this study, we report that Wnt signaling is required for two different aspects of monoaminergic circuit organization. First, Wnt signaling controls the A-P guidance of monoaminergic axons. Second, proper Wnt signaling is also required for the correct cell body orientation of both 5HT and mdDA neurons in the brainstem, which is disrupted in *Fzd3* mutant mice. We found that, at least for 5HT neurons, axon guidance appears to precede the final orientation of the soma. In wildtype animals, cell bodies of migrating, descending 5HT neurons initially point laterally but begin to re-orient along the A-P axis as they approach their final lateral position, thereby obeying the direction of their axon projections (Fig. 3N). Recent work by Hawthorne and colleagues uncovers similar mechanisms and indicates that early-differentiated 5HT neurons migrate ventrally from the ventricular zone to the pial surface by somal translocation (Hawthorne et al.). During somal translocation, neurons maintain a bipolar morphology and the soma translocates along primitive ventricular and pial processes. The lateral migration of descending 5HT neurons observed in our “open-book” preparations is reminiscent of somal translocation, with the axon being the leading process through which the cell body translocates. This would suggest that the direction of the axon projections dictate cell body orientation. However, one cannot exclude the possibility that cell body orientation and thus dendrite orientation may also be regulated independently from the axons by Wnt/PCP signaling. Future studies combining time-lapse imaging (Hawthorne et al.), fluorescently-labeled mouse lines (e.g. ePet-EYFP

lines (Scott et al., 2005)) and our newly developed “open-book” cultures will be required to further unveil the specific mechanisms underlying cell body re-orientation.

MdDA neurons are born in the ventricular zone of the mesodiencephalon and migrate ventrally along the processes of radial glia to ultimately form the SN and VTA (Smidt and Burbach, 2007; Tang et al., 2009). In PCP mutants, ventral (radial) migration of mdDA neurons is intact but in the most distal part of this migratory process the orientation of mdDA cell bodies that will eventually occupy more lateral domains (e.g. the SN) is perturbed (data not shown). One model to explain these defects, based on our observations in the descending 5HT system, is that the final stage of mdDA neuron migration, but not the radial migration, is guided by PCP signaling. Similar to what we suggest for the more laterally located descending 5HT neurons, PCP signaling may indirectly influence mdDA neuron cell body orientation by controlling axon guidance. Future experiments are needed to address these and other possibilities.

Concluding remarks

Here we show that Wnt/PCP signaling determines the proper A-P organization of ascending and descending serotonergic and dopaminergic pathways in the brainstem. Although Wnts had previously been reported to regulate the generation and differentiation of mdDA neurons (e.g. (Andersson et al., 2008)), our work establishes that these proteins are re-used as guidance cues for monoaminergic axons at subsequent developmental stages. This finding unveils a previously poorly characterized aspect of mdDA pathway formation and identifies one of the first guidance mechanisms involved in 5HT circuitry formation *in vivo*. Furthermore, this is, to our knowledge, the first example of a role for Wnt/PCP signaling in A-P axon guidance outside the spinal cord.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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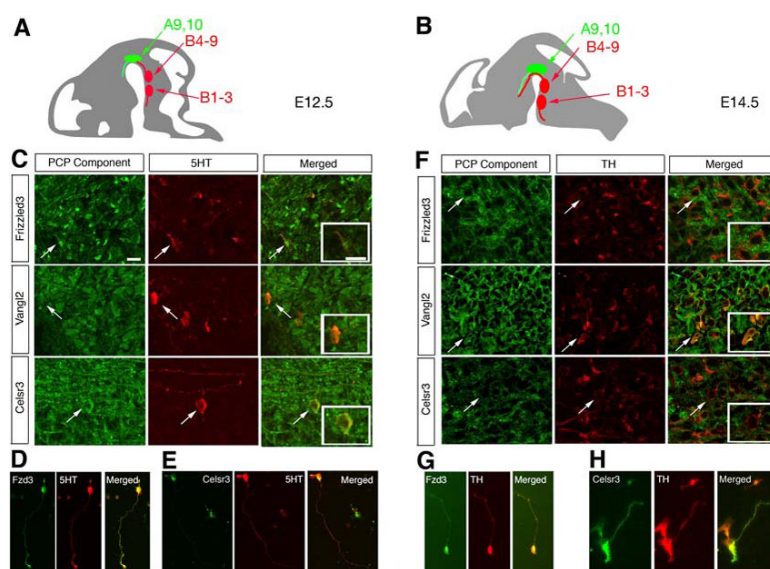


Figure 1. PCP signaling components are expressed in 5HT and TH positive neurons during midbrain and hindbrain development

Schematics in **A** and **B** indicate the anatomical location of the descending and ascending serotonin systems (B1-B3 and B4-B9; red) and the mesodiencephalic dopamine system (A9, A10; green) in mouse at E12.5 and E14.5, respectively. **(C)** Immunohistochemistry for 5-hydroxytryptamine (5HT) in red and PCP receptors in green in sagittal sections through the embryonic hindbrain at E12.5. Frizzled3, Vangl2, and Celsr3 all co-localize with 5HT positive neurons and fibers. Insets depict higher magnification images of cells indicated by the arrow. **(D)** Confocal images showing immunohistochemistry for tyrosine hydroxylase (TH) in red and PCP receptors in green in coronal sections through the E14.5 midbrain. Frizzled3, Vangl2, and Celsr3 all co-localize with TH positive neurons and fibers. Insets depict higher magnification images of cells indicated by the arrow. **(D, E)** 5HT+ dissociated neurons co-immunostained with 5HT in red and Fzd3 **(D)** or Celsr3 **(E)** in green. **(G, H)** TH + dissociated neurons co-immunostained with TH in red and Fzd3 **(G)** or Celsr3 **(H)** in green. Scale bar = 20 μ m.

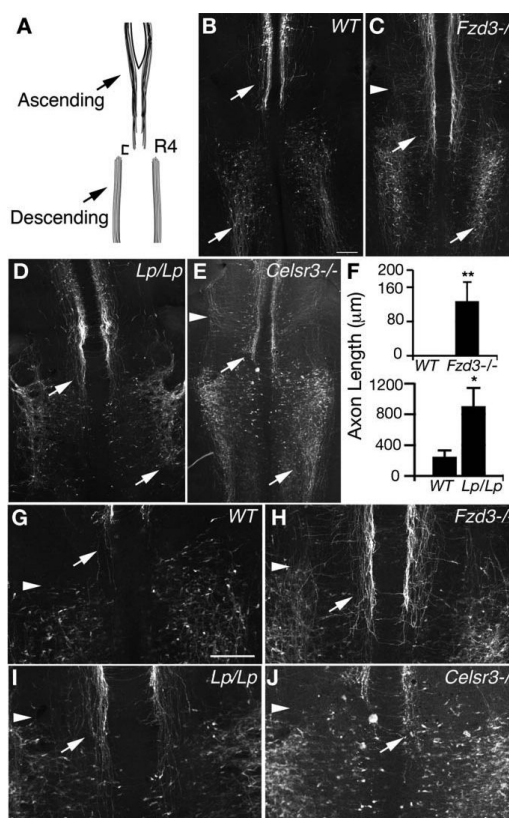


Figure 2. PCP signaling is required for axonal pathfinding of hindbrain serotonergic neurons (A) Schematic representation of the developing 5HT system at E12.5. (B-E) Whole mount immunostaining against 5HT in the hindbrain. At E12.5, ascending 5HT neurons in *Frizzled3* (*Fzd3*)^{-/-} (C), *Looptail* (*Lp/Lp*) (D), and *Celsr3*^{-/-} (E) mice all display axon misprojections into rhombomere 4 (R4) (see upper arrows). *Fzd3*^{-/-} and *Celsr3*^{-/-} also display laterally projecting axons within the ascending population (arrowheads in C, E). In addition, *Fzd3*^{-/-} (C) and *Lp/Lp* (D) mice display aberrant anteriorly projecting 5HT projections in the descending population and a marked reduction of proper descending axons (lower arrows). (F) Length of axon invasion into R4 in the *Fzd3*^{-/-} and *Lp/Lp* mouse. (G-J) Higher magnification of premature R4 invasion. Arrowheads indicate the start of the descending population and the arrow label axons from the ascending population. Scale bar = 250 μm.

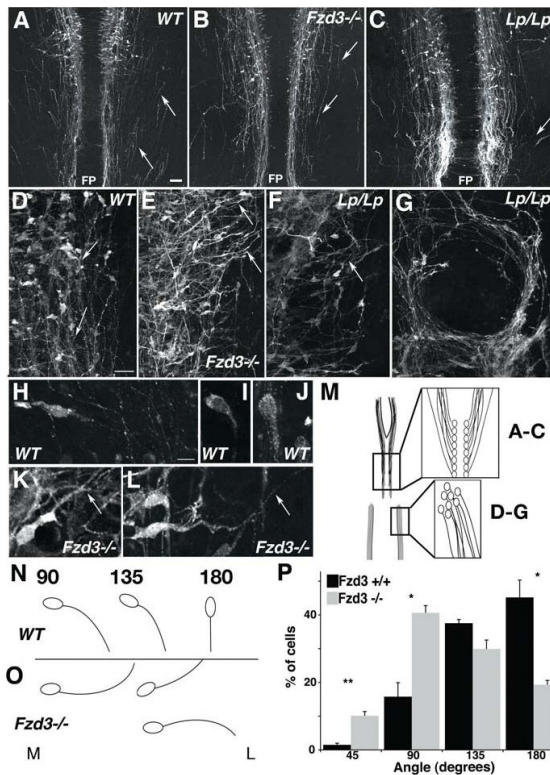


Figure 3. PCP mutant mice display specific axon guidance and cell body orientation defects in the 5HT system

(A-L) Whole mount 5HT immunostaining. Ascending 5HT populations are depicted in A-C and descending 5HT axons are shown in D-L. This is schematized in M. (A) In E12.5 wildtype (WT) embryos, axons in the ascending 5HT population emanate from the floor plate (FP) and strictly grow anterior towards the brain (arrows). In contrast, *Fzd3*^{-/-} (B) or *Lp/Lp* (C) mice display ascending axons that grow laterally and posteriorly (arrows). *Lp/Lp* mice display many “wavy” axons characteristic of axons lacking appropriate guidance. (D) In wildtype mice, descending axons project posteriorly towards the spinal cord at E12.5 (arrows). Both *Fzd3*^{-/-} (E) and *Lp/Lp* (F, G) mice display aberrant descending projections (arrows). *Fzd3*^{-/-} mice form very short axons within the descending region many of which project into inappropriate lateral and anterior directions (E). (G) *Lp/Lp* mice form axons within the descending region that display a clear fasciculation defects and grow in large circles. Cell body orientation is also affected in *Fzd3*^{-/-} mice. While during their mediolateral migration most WT descending neurons display cell bodies oriented first laterally (en route to their final position) and then along the anterior/posterior (A-P) axis as they reach their final lateral position (H-J), cell bodies in *Fzd3*^{-/-} mice are oriented in several different directions, many projecting at an acute angle near the midline and failing to orient posteriorly as they reach their lateral resting position (K, L). The cell orientation phenotype is schematized in (N, O) and quantified in (P). Graph shows average $\pm$ SEM. Almost 50% of *Fzd3*^{+/+} mice are oriented at 180° compared to *Fzd3*^{-/-} mice where only about 10% of neurons are oriented posteriorly. M, medial; L, lateral. Scale bar = 50 μ m (A-C) and 20 μ m (D-G), **P* < 0.05, ***P* < 0.01. (Number of mutants analyzed (*Fzd3*^{-/-}; n=3 (E11.5), n=15 (E12.5), n=2 (E13.5), *Celsr3*^{-/-}; n=3 (E11.5), n=4 (E12.5), *Lp/Lp*; n=4 (E12.5)).

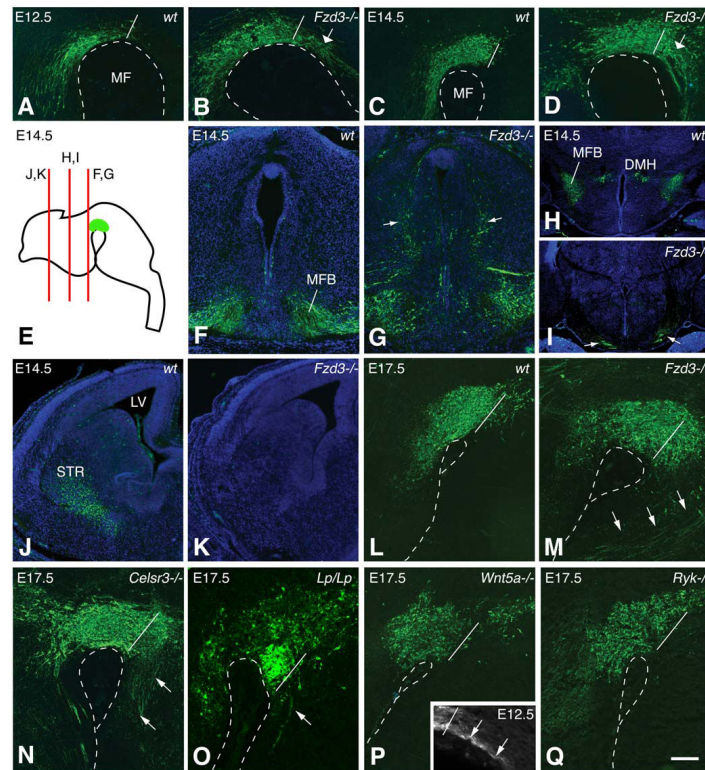


Figure 4. PCP mutant mice display axon guidance defects in the mdDA system

Immunohistochemistry for tyrosine hydroxylase (TH, in green), was used to analyze mdDA axon projections in *Fzd3*^{-/-}, *Celsr3*^{-/-}, *Lp/Lp*, *Wnt5a*^{-/-}, and *Ryk*^{-/-} mice and littermate controls at E12.5 (A, B), E14.5 (C-K), and E17.5 (L-Q). Coronal sections shown in panel F-K are counterstained with fluorescent Nissl to demarcate anatomical features. A-D and L-Q show sagittal sections. Whereas in wildtype (wt) control mice, mdDA neurons project their axons anteriorly towards the forebrain, numerous TH positive axons projecting posteriorly into the hindbrain region are found in *Fzd3*^{-/-} mice at E12.5, E14.5 and E17.5 (A-D, L, M, arrows indicate posterior misprojections). Dotted line outlines mesencephalic flexure (MF) and white line the posterior boundary of the mdDA system. Schematic in panel E indicates the location of sections shown in F-K. The medial forebrain bundle (MFB) is severely disrupted in *Fzd3*^{-/-} mice and ectopic axons can be found at dorsal levels in the brainstem but also near the midline (F, G) and in abnormal ventral regions in the developing hypothalamus (DMH) near the optic chiasm (H, I). In addition, TH positive axons fail to reach their synaptic targets such as the striatum (J, K) in *Fzd3*^{-/-} mice. *Celsr3*^{-/-} mice show a massive posterior back projection of TH positive axons at E17.5 (N). *Lp/Lp* mice also show posterior projections, but this defect is less pronounced as compared to *Fzd3*^{-/-} and *Celsr3*^{-/-} (O). In *Wnt5a*^{-/-} mice, a small number of axons project posteriorly at E12.5 (inset, Fig. S4) but at E17.5 this aberrant projection is no longer observed (P). *Ryk*^{-/-} mice show normal TH positive axon projections (Q). (Number of mutants analyzed: *Fzd3*^{-/-}; n=13 (E12.5), n=19 (E14.5), n=8 (E17.5), *Celsr3*^{-/-}; n=4 (E17.5), *Lp/Lp*; n=2 (E17.5), *Wnt5a*^{-/-}; n=4 (E17.5), *Ryk*^{-/-}; n=3 (E17.5)). Scale bar = 100 μm (A-D, F-K), 50 μm (L-Q).

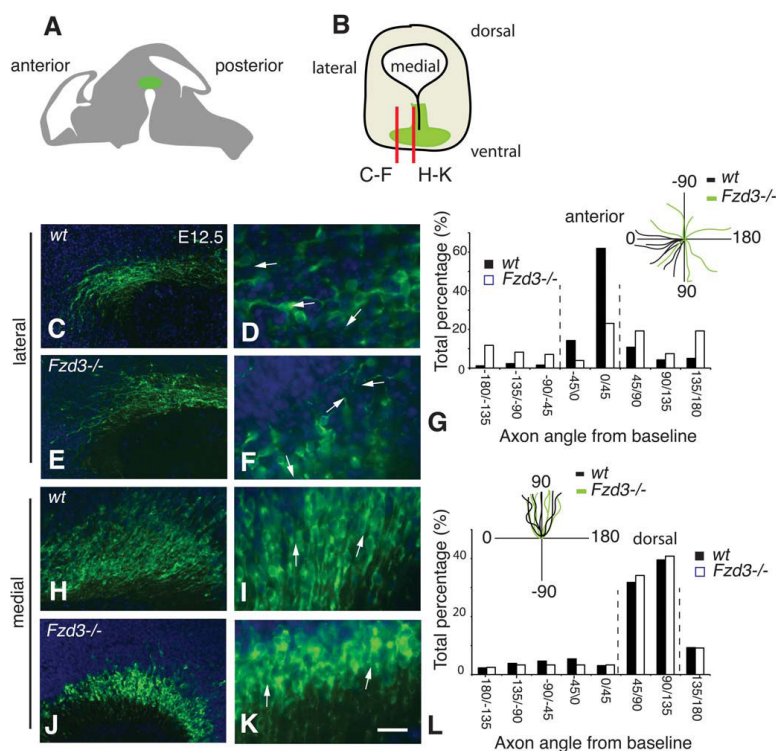


Figure 5. mdDA cell body orientation defects in *Fzd3* mutant mice

(A, B) Sagittal and coronal view of the mdDA system (green). (B) Red lines indicate the location of panels C-F and H-K. (C-F, H-K) Immunohistochemistry for tyrosine hydroxylase (TH, green) of E12.5 sagittal sections. Sections are counterstained with DAPI in blue. In wildtype mice, most TH positive neurons in the lateral mdDA system project their axons anteriorly (C, D, arrows), while medially located neurons project dorsally (and then anteriorly) (H, I). In contrast, in *Fzd3*^{-/-} mutants TH positive neurons at lateral levels project their axons randomly, i.e. no longer confined to their normal anterior direction (E, F). This effect is quantified as shown in G. (J, K) Neuronal orientation is largely intact at medial levels in *Fzd3*^{-/-} mutants. This is quantified as shown in L. Quantifications shown in G and L represent angles between the lining of the mesencephalic flexure (baseline, represents 0°) and the trajectory of the axon segment just proximal to the cell body. Inserts show axon orientation plots for a few randomly selected axons. Scale bar = 25 μm (C, E, H, J), 12.5 μm (D, F, I, K).

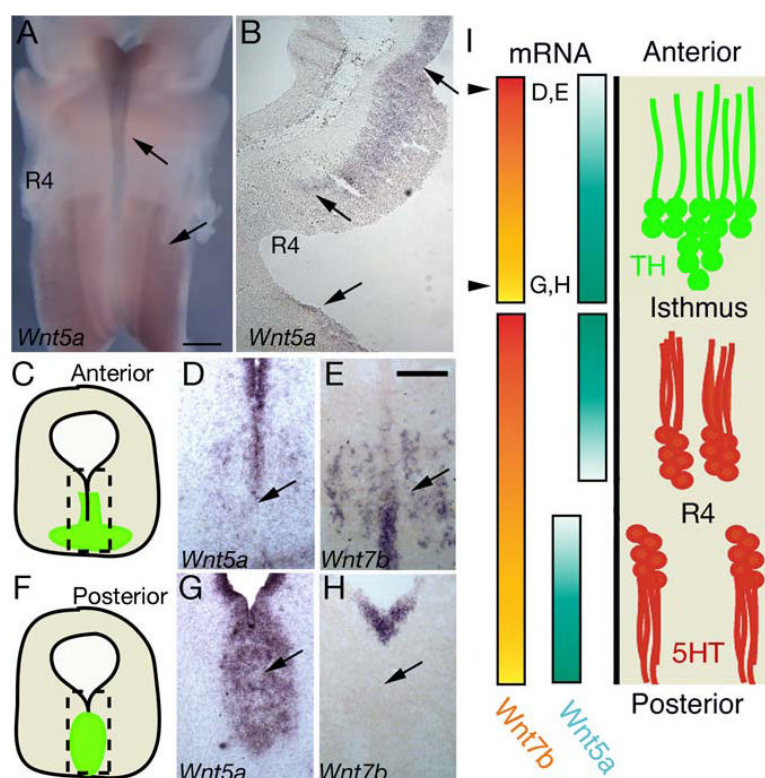


Figure 6. Multiple Wnts are expressed in gradients in both the hindbrain and midbrain
(A) Whole mount *in situ* hybridization for *Wnt5a* in E12.5 mouse embryos. Arrows mark the locale of both ascending and descending 5HT populations. **(B)** *In situ* hybridization for *Wnt5a* in a sagittal section of E12.5 hindbrain showing a anterior high, posterior low gradient near the midline and below the isthmus consistent with the location of pathfinding ascending 5HT axons at E12.5. **(C, F)** Schematics indicating the location of images shown in **D-H**. *In situ* hybridization for *Wnt5a* and *Wnt7b* in coronal **(D-H)** cryosections of the E14.5 midbrain region reveals a high posterior, low anterior expression gradient for *Wnt5a* and a high anterior, low posterior gradient for *Wnt7b*. **(I)** Proposed model for the role of Wnt/PCP signaling during A-P guidance of 5HT and TH axons. 5HT neurons (red) express the PCP components Fzd3, Celsr3 and Vangl2 to sense an attractive *Wnt5a* expression gradient in the hindbrain region directing ascending 5HT axons anteriorly and descending 5HT axons posteriorly. TH neurons (green) sense both an attractive *Wnt7b* gradient and repulsive *Wnt5a* gradient guiding their axons anteriorly.

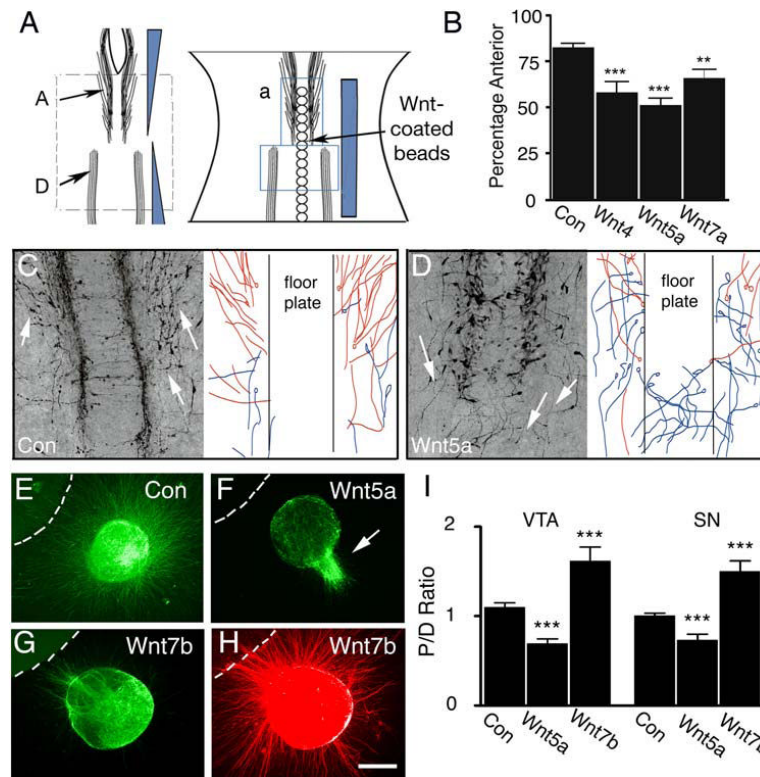


Figure 7. Both hindbrain serotonergic fibers and midbrain dopaminergic axons respond to Wnt proteins

(A) Wnt coated beads were placed at the midline of E12.5 mouse hindbrain “open book” preparations, cultured for 24 hrs and subjected to 5HT whole mount staining. (C, D) Addition of Wnt-coated beads to the midline disrupts the number of axons that project anteriorly in the ascending region. Graph in B shows average of different experiments $\pm$ SEM. (Control $n=10$, Wnt4 $n=6$, Wnt5a $n=10$, Wnt7a $n=9$), *** $p<.001$, ** $p<.05$ Student's T test. (C) Representative pictures of hindbrain explants showing the ascending 5HT system as revealed by 5HT immunostaining. Axons in control, H₂O coated beads, project anteriorly (arrows, C) while the presence of Wnt4, Wnt5a, and Wnt7a coated beads at the midline alters the appropriate location and projections of these neurons. Drawings adjacent to the immunostainings in C and D are schematic representations of anteriorly (red) and posteriorly (blue) projecting 5HT axons. (E-H) E12.5 VTA explants cultured adjacent to HEK293 cells (dotted line) secreting either control protein (E; $n=26$), Wnt5a (F; $n=12$) or Wnt7b (G, H; $n=10$). Explants are co-immunostained for TH (green) and β III-tubulin (red). (I) Quantification of the length of TH positive neurites in the proximal (P) and distal (D) quadrants of culture assays as shown in E-H. Graph shows average P/D ratio $\pm$ SEM. Note that whereas Wnt5A repels TH positive axons emanating from SN and VTA explants, Wnt7b attracts TH positive axons (SN explants: con, $n=9$; Wnt5a, $n=9$; Wnt7b, $n=9$ For each experimental condition, at least three independent experiments were performed. Scale bar = 100 μ m (B, D), 50 μ m (C, E), 80 μ m (F-I). *** $P<0.001$, one-way ANOVA.

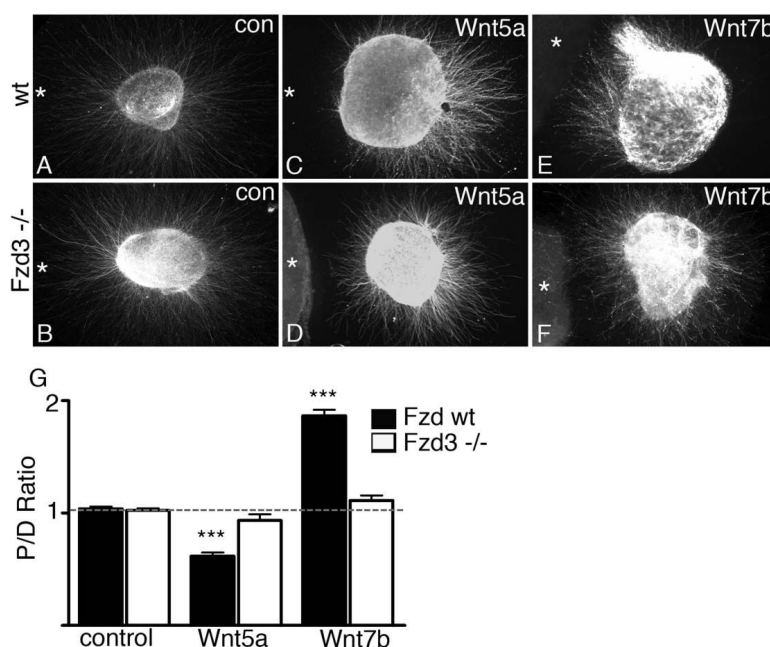


Figure 8. Dopaminergic neurons from *Fzd3* mutant mice are unresponsive to Wnt5a and Wnt7b E12.5-E14.5 VTA explants from either wild-type (**A, C, E**) or *Frizzled3* mutant (**B, D, F**) mice were cultured adjacent to HEK293 cells (asterisks) secreting either control protein (**A, B**; $n=19$ (for wt and KO), Wnt5a (**C, D**; $n=16$ (wt), 18 (KO)), or Wnt7b (**E, F**; $n=17$ (wt), 18 (KO)). Explants are immunostained for TH. (**G**) Quantification of the length of TH positive neurites in the proximal and distal quadrants of culture assays. Graph shows average P/D ratio $\pm$ SEM. Dopaminergic axons from wild-type but not *Fzd3* mutant mice are repelled by Wnt5a and attracted by Wnt7b. For each experimental condition, at least three independent experiments were performed. Number of mice analyzed: *Fzd3*^{-/-}; $n=6$, *Fzd3*^{+/+}; $n=8$. Scale bar = 80 μ m. *** $P<0.001$ as compared to control, one-way ANOVA. No statistically significant differences were found between KO-Wnt5a and WT-con or KO-con or between KO-Wnt7b and WT-con or KO-con.

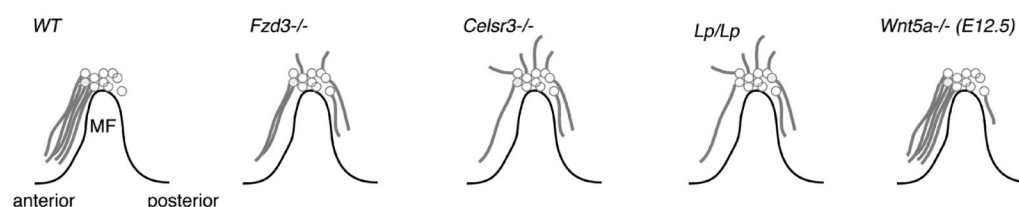
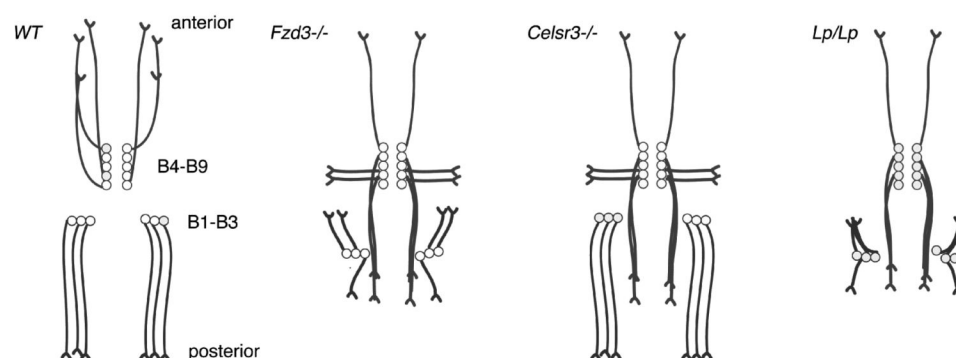
A Dopaminergic system**B** Serotonergic system

Figure 9. Axon guidance defects in the brainstem region of PCP and Wnt5a mutant mice
 Schematic representation of the embryonic meso-diencephalic dopamine (mdDA) system (**A**, sagittal view) and ascending and descending serotonergic systems (**B**, horizontal view). (**A**) In wildtype (WT) mice, mdDA axon projections are oriented anteriorly. *Frizzled3*, *Celsr3* and *Vangl2* (*Lp/Lp*) mutant mice display aberrant dorsal and caudal projections (from E12.5 until E17.5, the latest timepoint examined). *Wnt5a* mutant mice display a transient abnormal caudal projection at E12.5. (**B**) In wildtype mice, ascending serotonergic neurons (B9-B4) project their axons anteriorly, while descending neurons (B3-B1) give rise to posteriorly directed axon projections. *Fzd3* and *Vangl2* (*Lp/Lp*) mutant mice show ascending neurons that project their axons posteriorly or laterally and descending neurons that project axons anteriorly. *Celsr3* mutant mice predominantly show defects in ascending axon projections. Descending projections in *Vangl2* mice show hyperfasciculation phenotypes not found in *Fzd3* or *Celsr3* mutant mice.